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Quality evaluation of golden saxifrage (*Chrysosplenium alternifolium* L.) through simultaneous determination of four bioactive flavonoids by high-performance liquid chromatography with PDA detection

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ABSTRACT

To control the quality of *Chrysosplenium alternifolium* L., a simple, fast and reliable method of high-performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA) was developed and validated for simultaneous quantitative determination of four bioactive polymethoxylated flavonoids, namely chrysosplenosides B and D, and chrysosplenols B and D. Separation of the four analytes was accomplished on a C18 Hypersil ODS column (5 μm, 125 mm × 4 mm, i.d.) with an acetonitrile 10–100% (v/v) elution gradient, recorded at 345 nm. The equilibration of the methanol extracts and standard solution to 30% (v/v) of water was found to be necessary when minimizing viscosity differences between injections and the mobile phase, and thereby when minimizing distortions of analyte peaks and maximizing the resolution of critical bands of chrysosplenosides B and D. The correlation coefficients of all the calibration curves showed excellent linearity ($r = 0.9999$) over the wide test range. The relative standard deviation of the method was less than 3.53 and 4.41% for intra- and inter-day assays, and the average recoveries were between 95.3 and 103.5%. High sensitivity was demonstrated with detection limits between 0.012 and 0.029 μg/ml (0.24–0.58 ng). *C. alternifolium* was found to be a valuable source of the flavonoids with the total content ranging from 2.456 to 4.314% of dry weight, depending on harvest time and cultivation area. The total flavonoids were also determined using the pharmacopeial UV-spectrophotometric method and a notable underestimation was found in comparison to the developed HPLC method.

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1. Introduction

The plant genus *Chrysosplenium* (Turn.) L. of the family Saxifragaceae comprises about 60 species of semi-aquatic perennial herbs that are natives of the north temperate zone and southern South America. One of these, *Chrysosplenium alternifolium* L. (alternate-leaved golden saxifrage), is most common in European and Polish flora [1,2]. *C. alternifolium* and some other *Chrysosplenium* species are used in traditional European and oriental medicines for symptomatic treatment of digestive disorders related to liver and spleen activity [3], dizziness [4], skin diseases [5], and potentially the common cold [6]. Raw young spring leaves are also used as food ingredients and eaten in salads [7].

Chemical and pharmacological surveys of the genus afforded several highly bioactive polymethoxylated flavonols, derivatives of 5-hydroxy-3-metoxyflavone [8,9], which have been reported to be potent and specific antiviral agents (inhibitors of the replication of human rhinoviruses) [6], antitumor [5,6,10] and antioxidant agents [11]. Four flavonoids of this type (Fig. 1), two glycosides and two aglycones (chrysosplenoside B, CDB; chrysosplenoside D, CDD; chrysosplenol B, CLB; and chrysosplenol D, CLD), have been isolated with high yields from *C. alternifolium* in our laboratory [12], and could be considered as the marker compounds for the standardization of the herb.

Plant medicines and food samples containing flavonoids are frequently standardized by the UV spectrophotometric assay, in which after acid hydrolysis the released flavonoid aglycones are complexed with aluminium chloride, as prescribed in pharmacopeias [13]. It is well known that this method is not accurate in many herbal drugs, mainly owing to significant differences in the molar extinction coefficients of analyzed aglycones and the reference quercetin [14]. Moreover, the hydrolysis stage in the UV method makes the profile studies of real plant metabolites impossible. These problems can be overcome by use of modern chromatographic techniques, such as high-performance liquid chromatography (HPLC) [15], high-performance thin-layer (HPTLC) densitometry [16], gas chromatography (GC) [17] or capillary electrophoresis (CE) [18].

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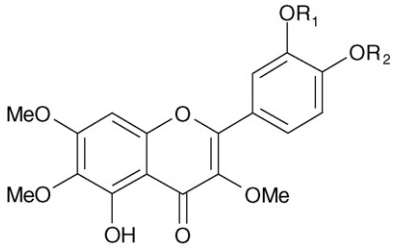

Fig. 1. Structures of four flavonoids found in *C. alternifolium*.

	R_1	R_2
Chrysosplenol B (CLB)	Me	H
Chrysosplenol D (CLD)	H	H
Chrysosplenoside B (CDB)	Me	β-glucopyranosyl
Chrysosplenoside D (CDD)	H	β-glucopyranosyl

However, no such method is currently available for separation of flavonoids in *C. alternifolium*. There was only one previous report for HPLC determination of flavonoids in tissue culture of *C. americanum* [19], but this species contained different flavonoid constituents, the conditions described allowed separation of flavonoid glycosides only, leaving aglycones coeluted, and therefore, the method is not suitable for quality control of *C. alternifolium*.

The percentage of components in plants, especially in herbs, may vary greatly with the growth environment and depends mainly on climate, region of cultivation and season of harvest [20]. In addition, as the different analytes, for example flavonoid glycosides and aglycones, have different chemical characteristic, the methods and solvents used for extraction will strongly influence the extraction yields and purities [21]. Besides the quality of separation, these factors are often crucial in the development of universal and accurate HPLC standardization procedures of herbal medicines [22,23].

Therefore, the present work attempted to optimize and validate a simple, accurate and sensitive method for extraction and simultaneous HPLC-PDA determination of CDD, CDB, CLD and CLB in samples of *C. alternifolium*. The proposed method can be readily used as a quality control tool and was successfully applied to analyze the above four flavonoids in samples of *C. alternifolium* from different harvest seasons and different cultivation areas in Poland. The results of HPLC assays were also compared to the classic UV-spectrophotometric method calibrated with CLD and CLB.

2. Experimental

2.1. Plant material and chemicals

The samples (aerial parts) of flowering *C. alternifolium* (Table 4) were collected at the end of April in five consecutive years (2004–2008) from two different climate areas located in central (province Łódź) and eastern Poland (province Białystok). Ten voucher specimens, identified and authenticated by Professor Jan Gudej, have been deposited in the Herbarium of the Department of Pharmacognosy, Medical University of Łódź, Poland.

HPLC-grade reagents and solvents used in HPLC assays were purchased from POCh, Poland (acetonitrile) and from Merck, Germany (water, orthophosphoric acid). The HPLC grade standard of quercetin (QU) used in the UV photometric assay was purchased from Sigma–Aldrich, Germany. The standards of four *Chrysosplenium* flavonoids (CDB, CDD, CLB, CLD) were previously isolated in our laboratory, and their structures have been confirmed by spectroscopic methods (UV, ^{1}H NMR) [12]. Before quantitative assays, the purity of the isolates was determined to be more than 95% by normalization of peak areas detected by HPLC-PDA.

All other reagents and solvents used in assays were of analytical-grade and were purchased from POCh, Poland.

2.2. Chromatographic analysis

2.2.1. Sample preparation

The samples were prepared by air-drying in normal conditions, powdering and sieving through a 0.315 mm sieve. An accurately weighed powder (300 mg) was extracted by refluxing with 30 ml of methanol for 30 min. After filtration, the sample was refluxed twice with 15 ml of methanol for 15 min. The combined extracts were diluted with 1 ml of chloroform to equilibrate the solvent composition of the injected extracts to that of the standard solutions (see Section 2.2.3), next with 30 ml of water, and finally with methanol to 100 ml. The diluted extracts were filtered through a PTFE syringe filter (13 mm, 0.2 μm, Whatman, UK), and the filtrate was directly injected (20 μl) into the HPLC system. Determinations were performed after three separate extractions of each sample, and each extract was injected at least in triplicate.

2.2.2. Instrumentation and conditions

The analyses were carried out on a Waters 600E Multisolvent Delivery System (Waters Co., MA, USA) with a photodiode array detector (PDA 996, Waters) working in the range of 190–800 nm, a 20 μl sample injector (Rheodyne 7725 i) and a LC workstation equipped with Waters Millenium 32 version 4.0 software for data collection and acquisition. A C18 Hypersil ODS column (5 μm, 125 mm × 4 mm, i.d.) (Agilent Technologies, CA, USA) guarded by a C18 Hypersil ODS pre-column (5 μm, 4 mm × 4 mm, i.d.) and maintained at room temperature was used. The mobile phase consisted of solvent A (0.5%, w/v solution of orthophosphoric acid in water) and solvent B (acetonitrile) with the elution profile as follows: 0–1 min, 10% B (isocratic elution, v/v); 1–3 min, 10–30% B (linear gradient, v/v); 3–7 min, 30% B (isocratic elution, v/v); 7–11 min, 30–85% B (linear gradient, v/v); 11–12 min, 85–100% B (linear gradient, v/v); 12–13 min, 100% B (isocratic elution, v/v); 13–14 min, 100–10% B (linear gradient, v/v); 14–20 min, 10% B (isocratic elution, v/v). Each run was followed by an equilibration period of 10 min. The detection wavelength was set at 345 nm. The flow rate was 1.0 ml/min. The analytes were identified by comparing their retention times and UV spectra with those of the standards. The peak purities were verified by PDA analysis of impurities.

2.2.3. Calibration, precision and accuracy

Validation of the developed method was carried out according to the ICH Guidance for Industry [24].

Four *Chrysosplenium* flavonoids were determined in plant samples by the external standard method. Because CLD and CLB are relatively hydrophobic compounds, the standards were first dissolved in 5 ml of chloroform–methanol (20:80, v/v), and next diluted with 65 ml of methanol and 30 ml of water to obtain the standard stock solution at the final concentrations of 97.20 μg/ml for CDB, 89.60 μg/ml for CDD, 38.80 μg/ml for CLB and 38.80 μg/ml for CLD, respectively. This standard solution was stored in the dark at 4 °C (the HPLC analysis indicated that this solution was stable for at least 3 months). Immediately before calibration the stock solution was diluted in triplicate with chloroform–methanol–water (1:69:30, v/v/v) to 70, 40, 10, 5, 1 and 0.1% of the starting concentration. The software Millenium (Waters) was used to fit the regression curves and to calculate the corresponding correlation coefficients (Table 1). All the dilutions were analyzed in triplicate.

The repeatability of the method was tested using measurements of intra- and inter-day variability. The precision was examined on real plant material samples (B 2005, L 2005) and also on standard solutions containing all four analytes at two different concentrations. The intra-day variability was determined by analyzing each

Table 1
Linearity of standard curves and detection/quantitation limits for the four flavonoid standards.

Analyte	Test range (μg/ml)	Calibration equation[a]	r^2[b]	LOD[c]		LOQ[c]	
				μg/ml	ng	μg/ml	ng
CDD	0.090–89.600	$y = 50970x + 7429.8$	0.9999	0.027	0.54	0.090	1.80
CDB	0.097–97.200	$y = 45761x + 5080.1$	0.9999	0.029	0.58	0.097	1.94
CLD	0.039–38.800	$y = 70379x + 833.35$	0.9999	0.012	0.24	0.039	0.78
CLB	0.039–38.800	$y = 64298x + 1919.5$	0.9999	0.012	0.24	0.039	0.78

[a] y = peak area, x = concentration of standards in μg/ml.
[b] r = correlation coefficient for seven data points ($n = 3$) in the calibration curves (linear model).
[c] LOD = limit of detection, LOQ = limit of quantitation.

Table 2
Retention parameters and precision data for the developed HPLC method.

Analyte	RT ± S.D. (min)[a]	k'[b]	Theoretical plates ($N \times 10^{-3}$)	Intra-day variability R.S.D. (%) for:				Inter-day variability R.S.D. (%) for:			
				Standard solution[c]		Real samples[d]		Standard solution[c]		Real samples[d]	
				$c_{100\%}$	$c_{1\%}$	L 2005	B 2005	$c_{100\%}$	$c_{1\%}$	L 2005	B 2005
CDD	9.720 ± 0.130	4.963	39.2	0.44	1.34	0.22	0.10	0.88	2.05	0.97	0.58
CDB	10.430 ± 0.158	5.399	34.7	0.22	0.59	0.20	0.15	0.74	1.54	0.83	0.67
CLD	12.584 ± 0.051	6.720	177.6	0.68	0.65	3.53	0.57	1.23	1.33	4.41	1.56
CLB	13.521 ± 0.035	7.295	172.6	0.39	0.39	0.57	0.36	1.27	1.52	1.23	1.29

[a] Mean values of retention times (RT) for standard solution tested for inter-day variability ($n = 50$), S.D. = standard deviation.
[b] Capacity factors (k') calculated with the hold-up time 1.630 ± 0.021 min.
[c] Values for the standard solutions tested at 100% ($c_{100\%}$) and 1% ($c_{1\%}$) of the stock concentration described in Section 2.2.3.
[d] Values for real samples (sample descriptions as in Table 4).

sample five times within 24 h, and the inter-day reproducibility was determined on five different days with each sample injected five times on each of the days. The relative standard deviation (R.S.D.) values were calculated for integration area and considered as a measure of precision (Table 2).

To evaluate accuracy, the recovery tests were carried out using extracts of two *Chrysosplenium* samples (B 2005, L 2005). The test extracts were prepared using double the mass of the plant material (600 mg) compared to the procedure described in Section 2.2.1, and three portions of 5 ml of the extract were transferred into three volumetric flasks. Two of them were spiked with 0.25 and 1 ml of the standard stock solution. Finally, all three extract samples were diluted with chloroform–methanol–water (1:69:30, v/v/v) to 10 ml. Five HPLC analyses were performed for each diluted sample. The accuracy was evaluated by calculating the mean recovery of the four flavonoids from the spiked extract solutions versus the non-spiked extract sample (Table 3).

2.3. Optimization of the addition of water to the extracts

The herb sample (B 2006, 350 mg) was extracted twice by refluxing for 30 min with 30 ml of methanol, and the extract was diluted with methanol to 100 ml. The 6 ml portions of the obtained solution were transferred in triplicate into five volumetric flasks, adjusted with different volumes (0–4 ml) of water, and finally diluted with methanol to 10 ml. Triplicate HPLC analyses were performed for each diluted sample. The influence of the addition of water on peak shape and the separation quality of the extract is presented in Fig. 4.

Table 3
Recovery of the four flavonoids in the extracts of *C. alternifolium*.

Analyte	Initial mean concentration in the extract[a] (μg/ml)	Amount added (μg/ml)	Concentration after addition[a] (μg/ml)		Recovery (%)	R.S.D.[b] (%)
			Expected	Measured		
CDD	33.69[c]	8.96	42.65	42.30	99.2	0.83
		2.24	35.93	35.31	98.3	1.25
	70.31[d]	8.96	79.27	82.05	103.5	1.12
		2.24	72.55	73.14	100.8	0.62
CDB	39.49[c]	9.72	49.21	48.91	99.4	0.70
		2.43	41.92	41.37	98.7	0.83
	46.69[d]	9.72	56.41	56.19	99.6	0.99
		2.43	49.12	48.04	97.8	1.24
CLD	0.27[c]	3.88	4.15	3.99	96.2	2.40
		0.97	1.24	1.18	95.3	2.49
	8.68[d]	3.88	12.56	12.22	97.3	1.13
		0.97	9.65	9.47	98.1	2.56
CLB	0.68[c]	3.88	4.56	4.38	96.2	2.34
		0.97	1.65	1.57	95.6	2.55
	5.92[d]	3.88	9.80	9.65	98.4	0.76
		0.97	6.89	6.67	96.7	1.87

[a] Mean concentration of the analyte in the final analytical solution ($n = 5$).
[b] R.S.D. values for $n = 5$.
[c] Concentrations measured in the extract of sample: L 2005 (sample descriptions as in Table 4).
[d] Concentrations measured in the extract of sample: B 2005 (sample descriptions as in Table 4).

2.4. Spectrophotometric analysis

The methodology used was based on the pharmacopoeial procedure described for determination of total flavonoid content in plant materials [13], with a slight modification: 400 mg of the powdered sample was used. Furthermore, three flavonoid aglycones were tested as calibration standards: QU, CLB and CLD. The quantitative data (Table 5) were obtained by plotting the concentrations of standards (μg/ml) versus absorbance measured using a Specol spectrophotometer (Carl Zeiss, Germany) in 10 mm cuvettes.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The effectiveness of HPLC separation was tested using both the standard solution and the methanolic extract from *C. alternifolium*, to allow for the possible interference of the sample matrix. The four flavonoids analyzed (Fig. 1) differ strongly in polarity, and therefore their separation required optimization of the gradient elution profile to obtain the highest resolution in the shortest analysis time. Initially, various proportions of acetonitrile–water were tested, because some polymethoxylated flavone aglycones that are closely related to *Chrysosplenium* flavonoids have recently been reported to be well resolved using this system [25,26]. As a result, the best separation was obtained with a 10–100% (v/v) acetonitrile gradient in an aqueous solution containing 0.5% (w/v) orthophosphoric acid and at the flow rate of 1 ml/min. However, in the case of peaks CDD and CDB we observed strong peak fronting and broadening (Fig. 3a). Peaks remained deformed with the use of methanol–water and tetrahydrofurane–water. Distortions of flavonoid peaks are usually caused by dissociation of the hydroxyl groups, and should be reduced by the presence of acid in the mobile phase, and thereby by changing the pH [27,28]. Unfortunately, neither different concentrations of orthophosphoric acid used nor the adjustment of the mobile phase with other acids, such as acetic acid or trifluoroacetic acid, resulted in an acceptable peak shape for CDD and CDB. Other factors known to cause peak distortions under RP-HPLC conditions are differences in solvent strength and viscosity between the sample solvent and mobile phase [29]. It is recognized that an injection solvent stronger than the mobile phase can interfere with the adsorption of the sample at the column head [30], while an injection of a pulse of different viscosity can produce flow instability known as viscous fingering [31,32]. The practice widely recommended to avoid these problems is to dissolve the samples to be injected in the mobile phase [29,30]. However, this method could not be applied to the *Chrysosplenium* extracts, because they are practically insoluble in the starting eluent (10% acetonitrile in water). Thus, to match the solvent strength and viscosity (η) between extracts dissolved in methanol ($\eta^{25^\circ C} = 0.545$ cP) and the starting mobile phase ($\eta^{25^\circ C} = 0.857$ cP), we proposed the addition of water ($\eta^{25^\circ C} = 0.891$ cP) to the crude extracts before injection. Fig. 4 displays the influence of the addition of water on the peak shape and separation of *Chrysosplenium* extracts. As shown, with the percentage of water increasing from 0 to 40%, and thereby with extract viscosity increasing from 0.545 to 0.731 cP, the peak heights increased, and the peak widths decreased. In consequence, the symmetry of all peaks of interest was significantly improved. Interestingly, with the addition of more than 20% water, the previously observed strong peak fronting (symmetry factor, $A_s < 1$) was replaced by slight tailing ($A_s > 1$). Although the changes in peak shape were observed for all four analytes, they were most distinctive for early eluting bands CDD and CDB, which confirmed that viscosity differences were the main cause of the described peak distortions [29]. As a result of improved peak shape, the resolution of critical bands CDD and CDB was also improved, and the resolution factor R_s, calculated based on peak widths at 5σ ($w_{5\sigma}$), increased from 1.74 to 3.09 as the percentage of water increased from 0 to 40%. However, extracts adjusted with more than 30% water exhibited some precipitation tendencies, probably due to the significant hydrophobicity of the aglycones CLD and CLB. Therefore, with the limit of high peak symmetry for all analytes ($1.12 \leq A_s \leq 1.16$, at 5% of the peak height) and an R_s value for adjacent bands CDD/CDB of 2.998, the adjustment of the extracts and standard solutions to 30% water was assumed to be optimum and it was applied in subsequent experiments.

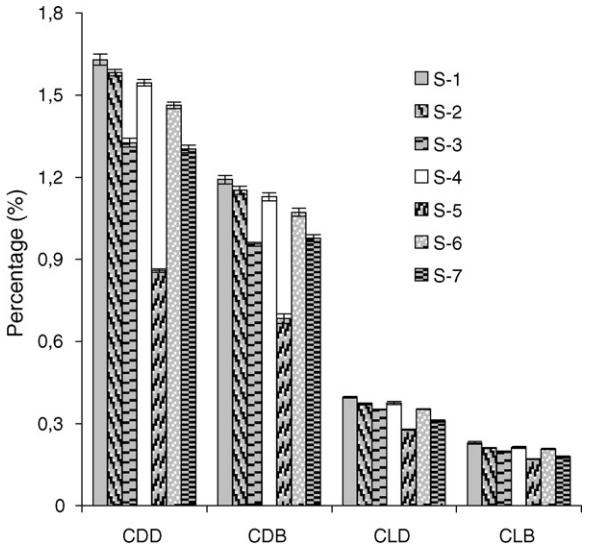

Fig. 2. Extraction efficiency of the four flavonoids from *C. alternifolium* (sample B 2006) in different solvent systems: S1, methanol; S2, methanol–water (80:20, v/v); S3, acetonitrile; S4, acetonitrile–water (80:20, v/v); S5, ethyl acetate; S6, chloroform–methanol (20:80, v/v); S7, acetone. Each column represents the mean ± S.D. of three independent extraction experiments. For extraction procedure see Section 3.2.

Selecting a proper detection wavelength is of great importance to ensure precise detection of the four *Chrysosplenium* analytes and to achieve the goal of maximizing absorption and minimizing interference. Due to the flavonol skeleton of the compounds [33], the UV–vis spectra acquired with the PDA detector for CDD, CDB, CLD and CLB dissolved in the mobile phase exhibited two major absorption bands, the first in the range of 250–270 nm, and the second in the range of 340–350 nm. For all four analytes, the absorption peaks in the second wavelength range were higher than the first peaks, and the absorption maxima were 340.9, 340.9, 350.5 and 349.3, respectively. Although with the PDA detector the individual compounds can be analyzed at different wavelengths, the average value 345 nm was chosen for the simultaneous determination of CDD, CDB, CLD and CLB to enable future applications of the developed method using simple HPLC-UV equipment.

3.2. Optimization of sample preparation

A key factor for accurate determination of plant metabolites is based on the extraction yield [22]. In order to determine the most convenient procedure for extraction of *Chrysosplenium* flavonoids, various solvents (Fig. 2), extraction periods (1–4) and extraction times (15, 30, 45, 60 min) were tested for reflux extraction of a herb sample. Reflux was chosen as the most effective extraction method based on previous optimization studies for some polymethoxylated flavonols [25] and polymethoxylated flavanone glycosides [22], which exhibit a similar polarity to the four *Chrysosplenium* flavonoids. The test extractants were chosen on account of a suitable

Table 4
Content of four flavonoids in the samples of *C. alternifolium* determined by the developed HPLC method.

Sample number[a]	CDD[b]		CDB[b]		CLD[b]		CLB[b]		Total
	%	R.S.D. (%)	%	R.S.D. (%)	%	R.S.D. (%)	%	R.S.D. (%)	%
B 2008	1.927	1.26	1.423	1.22	0.017	3.55	0.028	3.17	3.396
L 2008	1.880	0.09	0.869	0.50	0.018	3.78	0.019	2.30	2.787
B 2007	1.725	0.37	1.364	0.48	0.296	0.57	0.219	0.44	3.604
L 2007	1.557	0.24	0.757	0.56	0.337	1.12	0.175	0.59	2.828
B 2006	1.621	0.27	1.191	0.38	0.396	0.60	0.230	0.54	3.439
L 2006	1.189	0.40	1.175	0.19	0.091	0.52	0.127	0.47	2.583
B 2005	2.305	0.62	1.531	0.75	0.285	0.89	0.194	0.72	4.314
L 2005	1.116	1.02	1.309	0.83	0.009	3.78	0.022	2.14	2.456
B 2004	2.092	1.28	1.449	1.27	0.037	3.31	0.042	2.47	3.621
L 2004	1.761	1.20	1.068	1.39	0.034	2.50	0.033	0.28	2.896

[a] Samples collected in different years from areas with natural population near Łódź (L) and Białystok (B).
[b] Mean percentage content calculated per dry weight of the plant material. R.S.D. for $n = 9$ (three extractions and three injections for each extraction).

polarity range. To prepare the test extracts, the herb sample (B 2006, 300 mg) was extracted twice by refluxing for 30 min with 30 ml of the appropriate solvent. The combined extracts were adjusted to 30% water, diluted with methanol to 100 ml and subjected to HPLC analysis. All extract samples were assayed in randomized order to minimize the effects of uncontrolled factors that may introduce bias to the measurements. On the basis of the peak-area responses, methanol was found to be the best solvent yielding the highest concentrations of the four analytes (Fig. 2). In the further optimization of extraction time when using methanol as the extractant, three successive extraction periods, 30, 15, and 15 min long, respectively, resulted in the best yield.

3.3. Method validation

The calibration results obtained under the optimized sample preparation and chromatographic conditions described above are summarized in Table 1. The four calibration curves exhibited excellent linear regressions of $r = 0.9999$, over the wide concentration ranges. The high sensitivity of the method was demonstrated with the low LOD (0.012–0.029 μg/ml, 0.24–0.58 ng) and LOQ (0.039–0.097 μg/ml, 0.78–1.94 ng) values. The LOQs were assumed to be the lowest determined linear range limits, because relative standard deviations (R.S.D.) measured for more diluted standards were higher than 15%, and thereby too high for precise quantitative measurements [34]. The LODs were calculated successively using the widely accepted LOD/LOQ proportion $3\sigma/10\sigma$. Despite the restrictive calculation assumption, the LOD and LOQ values obtained were similar or even lower than those reported earlier for determinations of related polymethoxylated flavonol aglycones from *Ammomum koenigii* (0.27–0.46 ng [25]) or polymethoxylated flavones in citrus juices (LOQ < 2 ng [35]). To prove that the chosen analytical procedure is practically capable of detecting and quantitating the analytes at the LODs and LOQs estimated from calibration standards, the extract of the real *Chrysosplenium* sample (B 2006) was diluted in five replicates to concentration levels equaling those of the LODs and LOQs and triplicate HPLC analyses were done for the obtained sub-samples. Well-defined chromatographic peaks were observed at the LODs, and the R.S.D. values of the measurements at the LOQs were in the range of 9.2–13.5% proving the validity of the determined method limitations [24].

The results shown in Tables 2 and 3 indicate that the developed analytical method was reproducible with good recovery and stability. The intra- and inter-day variation, assayed at different concentrations of all analytes in standard solution and real extract samples, was less than 1.34 and 2.05%, respectively. Higher values (3.53 and 4.41%, respectively) were observed only for CLD detected in the real sample (L 2005) at trace concentration level (0.268 μg/ml; 0.009% dw).

The average recoveries, determined during the standard addition procedure, were satisfactory for all four flavonoids studied, with values ranging from 95.3 to 103.5%, thereby confirming the accuracy and robustness of the developed method.

3.4. Sample analysis

Ten samples of *C. alternifolium* collected in five consecutive years from two different climate areas in Poland were extracted and separated under the optimized conditions described above. The typical HPLC-UV profile at 345 nm is illustrated in Fig. 3b. The content of

Table 5
Comparative results of HPLC and UV-photometric assays for total content of flavonoid aglycones in *C. alternifolium*.

Sample number[a]	HPLC (CLD + CLB)[b] λ = 340 nm		UV (QU)[c] λ = 425 nm		UV (CLD)[d] λ = 405 nm		UV (CLB)[e] λ = 400 nm	
	%	R.S.D. (%)	%	R.S.D. (%)	%	R.S.D. (%)	%	R.S.D. (%)
B 2008	2.368	1.27	0.775	1.05	1.537	1.14	1.795	2.16
L 2008	1.941	0.18	0.704	1.35	1.455	2.12	1.644	3.06
B 2007	2.657	0.27	0.851	1.12	1.833	2.01	2.155	4.20
L 2007	2.115	0.23	0.773	1.08	1.542	1.70	1.786	2.30
B 2006	2.576	0.22	0.844	1.41	1.795	2.94	1.973	4.08
L 2006	1.859	0.25	0.668	1.00	1.327	1.45	1.391	2.38
B 2005	3.136	0.67	0.882	2.05	2.141	2.91	2.477	5.07
L 2005	1.714	0.80	0.641	0.68	1.205	1.18	1.326	1.34
B 2004	2.533	1.32	0.803	1.33	1.641	3.04	1.887	4.24
L 2004	2.027	1.19	0.727	1.56	1.436	2.72	1.677	3.08

[a] For sample description see Table 4. [b] The conversion factors for calculation of total aglycones [$f_1 = 0.670$ for the glycoside CDD (MW 522) and $f_2 = 0.698$ for CDB (MW 536)] were determined with the molecular weight of the aglycones CLD (MW 360) and CLB (MW 374). Calculation: total aglycones = CLD + CLB + ($f_1 \times$ CDD) + ($f_2 \times$ CDB), where CDD, CDB, CLD and CLB are the contents determined by the developed HPLC method (Table 4). [c–e] Total contents of aglycones determined by spectrophotometric assay [13] and calculated using linear calibration equations of QU ($y = 0.0656x + 0.0018$, $r = 0.9999$, test range: 1.04–13.52 μg/ml), CLD ($y = 0.0286x + 0.0151$, $r = 0.9985$, test range: 2.56–25.62 μg/ml) and CLB ($y = 0.0237x + 0.0203$, $r = 0.9997$, test range: 2.74–27.36 μg/ml). Calibration was performed by plotting the concentrations of standards (x: μg/ml) versus absorbance (y).

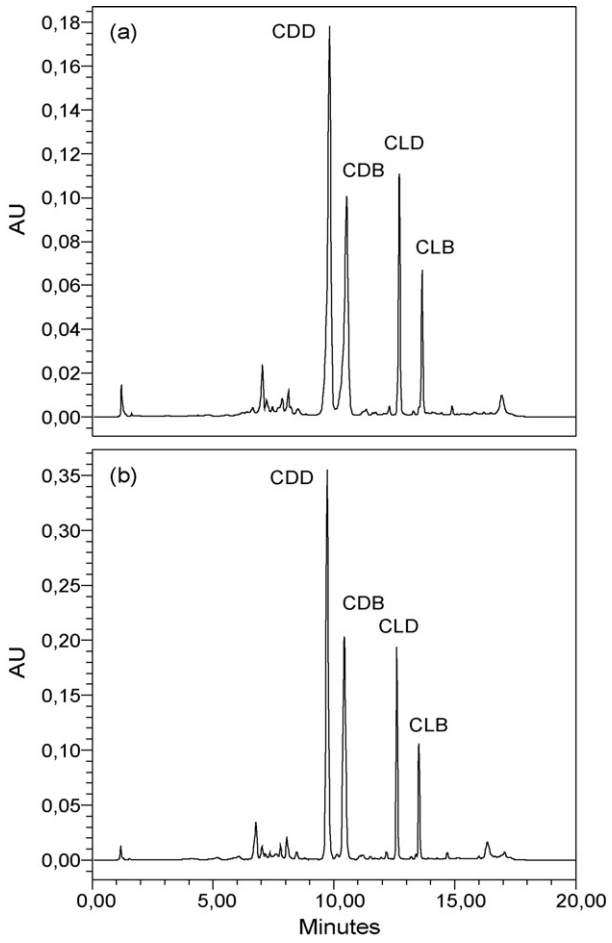

Fig. 3. HPLC chromatograms of the methanol extract from *C. alternifolium* (B 2006): (a) adding no water to the extract prepared according to Section 2.3; (b) obtained under optimized sample preparation procedure (see Section 2.2.1), CDD: 49.61 μg/ml, CDB: 36.45 μg/ml, CLD: 12.12 μg/ml, CLB: 7.04 μg/ml. Detection at 345 nm. For chromatographic conditions see Section 2.2.2.

each flavonoid in the samples was determined by the corresponding regression equation and the results are summarized in Table 4. The total content of flavonoids varied among the assayed samples in the range of 2.456–4.314% of the plant material dry weight (dw). Higher flavonoid levels (3.396–4.314% dw) were found in the samples collected in eastern Poland (near Białystok) than in those harvested in the center of the country (near Łódź, 2.456–2.896% dw). The R.S.D. value of the average flavonoid content was 18.12% for all samples, and only 10.09 and 6.78% for the groups of samples collected in the same province. This suggests that harvest area is the most important factor determining the flavonoid concentration in *C. alternifolium*, but more detailed studies, involving other growth locations and herb batches, should be performed to confirm this suggestion. Among the four analyzed flavonoids, the glycosides CDD and CDB were the dominant components in all samples with the total level ranging from 2.315 to 3.541% dw and constituting 81.87–98.72% of the total flavonoid level.

In view of the fact that UV-spectrophotometric methods are more accessible and economic and are still recommended by pharmacopeias, a comparison was made between the HPLC total flavonoids and the UV method used by the European Pharmacopoeia [13], using *C. alternifolium*. Calibration of the UV method was performed using QU, CLD and CLB as the aglycones standard. After complexation with aluminium chloride in acidified media, the absorbances were measured at three different wavelengths (425, 405 and 400 nm) corresponding to the different absorption

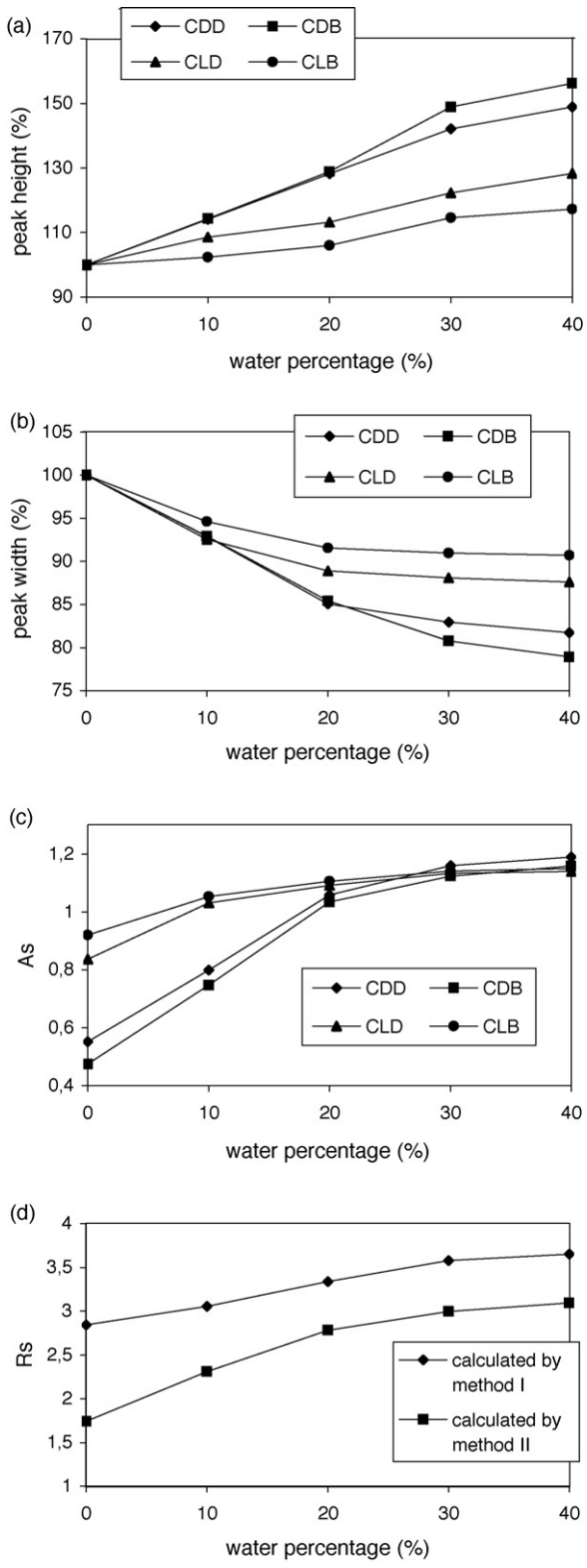

Fig. 4. Effect of addition of water to the extract on the peak heights (a), half height peak widths (b), symmetry factor A_s (c), and resolution factor R_s (c) of the analytes. All graph points are the mean of nine analyses. R_s was calculated for adjacent bands CDD/CDB by method I (based on half height peak widths, w_h) or method II (based on peak widths at 5σ, $w_{5\sigma}$), according to Ref. [36].

maxima of the appropriate complexes of the standards [12,33]. The results obtained (Table 5) indicated an underestimation of the total flavonoid content, but showed good correlation with the HPLC method ($r \geq 0.9579$). Extremely strong underestimation (60–70%) was observed with QU as a standard, which can be attributed mainly to the differences in absorption maxima and calibration slopes (a) between QU ($a = 0.0656$), CLD and CLB ($a = 0.0286$ or 0.0237). The results obtained with CLD and CLB as standards were better, but also lower by 25–36% or 16–26% (depending on the standard), compared to the HPLC total flavonoids. The differences may be related to the extraction error, due to the similar loss of CLD and CLB (22–23%) found during extraction experiments with acetone in comparison to methanol (Fig. 2); the solvents which were used as extractants in UV-spectrophotometric and in the developed HPLC method, respectively.

4. Conclusions

The RP-HPLC-PDA method reported here represents a simple, accurate and rapid technique for the simultaneous determination of four polymethoxylated flavonols in the samples of *C. alternifolium*. The assay is reproducible, highly sensitive, fully validated and was successfully applied to several herb samples collected from different growth areas and years of harvest. This work is the first report regarding flavonoid content in *C. alternifolium*, and the second report on the flavonoid content in the whole genus *Chrysosplenium* [19]. Given the known bioactivity of *Chrysosplenium* flavonoids [5,6,10,11] and the high levels found in the analyzed samples, the results obtained could explain some of the ethnomedical properties of the herb and rationalize its further medicinal use.

The popular UV-spectrophometric assay strongly underestimated the total flavonoid content in relation to the developed HPLC method, and therefore could not be recommended for the quality control of *C. alternifolium*. However, as a last resort, the UV method may be applied on condition that CLB is used as a calibration standard to minimize underestimation.

It is obvious that the use of short RP-HPLC columns and gradient elution procedures can significantly reduce the analysis time of multicomponent plant extracts. However, when the pre-treatment of a given sample ends with the analytes dissolved in a solvent different from that used in the starting eluent, several serious peak distortions may occur. The strategy recommended here, with adjustment of the sample with water before injection, is a very simple and fast procedure for preventing these problems, increasing the resolution, sensitivity and overall quality of the HPLC method used.

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